Understanding Variables that Impact Plasma Carotenoid Stability during Storage for High Performance Liquid Chromotography (HPLC) Analysis

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Submitted: April 19, 2017

ABSTRACT

Carotenoids are pigments ubiquitous in fruits and vegetables and noted as bioactive compounds affecting health. Understanding the relationship between carotenoids and health relies on precise and accurate analytical methods. The goal of the project was to understand factors that affect the stability of carotenoids from human plasma. If samples could be stored for a longer period of time, there would be more flexibility to extract samples, store and analyze in batches without fear of carotenoid degradation. Human plasma was obtained and tested for initial levels of carotenoids. The plasma was aliquoted, frozen, thawed and extracted according to a previously published procedure. The fat-soluble fraction was dried under nitrogen gas and stored in glass vials at 4°C, -20°C, and -80°C and stored for total of 120 days. The samples were removed, redissolved in methyl *tert*-butyl ether and methanol, and analyzed using HPLC, all in triplicate. Carotenoid levels (lutein, zeaxanthin, beta-cryptoxanthin, alpha-carotene, beta-carotene, other-*cis*-lycopene, all-*trans*-lycopene, 5-*cis*-lycopene, total lycopene, and total carotenoids) were quantitated at each time point and compared using two-way ANOVA and Tukey's posthoc test, with significance at $P<0.05$. Storage time had a significant effect for all compounds, and the interaction between the storage time and temperature was also significant for several carotenoids. However, the carotenoids were much more stable during storage, especially in the refrigerator, than expected. This information will be useful for future studies on carotenoids, depending on the compounds of interest, by optimizing storage conditions and efficiency, while preserving key compounds.

INTRODUCTION

Personalized nutrition is being researched as a way to customize a diet and lifestyle specific to one's genes and biochemistry. Carotenoids are bioactive compounds found in a variety of fruit and vegetables and are an important class of molecules to study. According to Campbell et al. (1994), carotenoids have been linked to reduced risk of disease and cancer, and can serve as a good marker of one's digestion and absorption of key nutrients. Carotenoids are antioxidants and thought to play a significant role in health maintenance (Khachik 1995). The most studied carotenoids in relation to human health are beta-carotene, lycopene, lutein, and zeaxanthin (Johnson 2002). However, they can also be somewhat unstable. They are sensitive to light and heat, but have been shown to be relatively stable in freezers for months (Craft et al 1988).

Carotenoids can be extracted from plants, foods, and biological samples to determine their concentration. In order to determine if carotenoids are exerting a biological effect, they must be able to be accurately measured in the body. It is common for carotenoids to be measured in blood plasma or serum. Factors after extraction but prior to analysis can potentially lead to some degradation of carotenoids, leading to inaccurate quantification carotenoids in the sample. Reversed phase high performance liquid chromatography (HPLC) is commonly used to separate carotenoids in plasma based on their affinity for a stationary phase. The column used interacts with compounds in varying strengths, so those with weaker interactions elute first. A solvent is pumped through the column under high pressure with a changing gradient to elute compounds of differing polarities. The amount eluted can be quantified by a photodiode array detector. A chromatograph can be generated with peaks plotting the absorbance of the elutent of the HPLC over time.

Standard procedures exist in sample handling prior to carotenoid analysis, but many factors that can affect quantification have not been investigated thoroughly. In a time when such analytical chemistry and personalized nutrition is a quickly growing field, the efficacy of the analysis is extremely important.

Accurate carotenoid analysis is critical for continued study to understand bioefficacy. Understanding what factors affect the extraction efficiency and stability of carotenoid extracts will inform the execution of their analysis. Many research projects depend on accurate extraction data and knowing the best procedure can ensure the best results. Storage time of extracted samples is necessary for large-scale projects because it would be extremely helpful to be able to extract carotenoids from plasma and then have several months to analyze them without worrying about decomposition. If samples are stable redissolved prior to analysis, it would allow the queuing of more samples at one time, increasing the number of samples that can be analyzed per day.

OBJECTIVES

The purpose of this project is to investigate variables of storage that may impact carotenoid stability from blood plasma. This includes the storage temperature and time. The results from this investigation will help future projects that involve carotenoid extraction and quantification, and will optimize protocols for the most accurate data. This study will highlight the most crucial points of the storage in which carotenoids are susceptible to degradation and isomerization. Overall, the purpose of this study is to find the answers to the decomposition questions in order to improve future studies.

METHODS

Human plasma was screened to find donor plasma that contained levels of carotenoids similar to those found in western populations. After screening 10 donors, the most appropriate was chosen and blood plasma was obtained (Interstate Blood Bank, Memphis, TN) and arrived chilled at 20°C. The plasma was immediately aliquoted into 1 mL vials and then frozen. For extraction, the plasma was thawed in cool

water until liquid, about 10-12 minutes. For all samples of this project, the carotenoids were extracted using the following procedure, as previously descibred (Barona 2012). Briefly, 1 mL of thawed plasma wascombined with 1 ml ethanol (with 0.1% butylatedhydroxytoulene) and 5 mL of HEAT (hexane/ethanol/acetone/toluene, 10:6:7:7) in an 11 mL vial. Then, the sample was probe sonicated for 8 seconds, and then centrifuged at 3,000 rpm for 2 minutes. The upper, nonpolar layer of the vial was removed using a syringe and placed into an 11mL vial. Another 5 mL of HEAT was added to the first vial; the mixture was sonicated and centrifuged again at the same conditions. The nonpolar layer was again removed and combined with the first extract removed. The vial were dried under nitrogen gas and stored at -80° C, -20° C, or 4° C until analysis. The storage time points were included 0, 1, 3, 7, 14, 21, 31, 56, 90, and 120 days. All time/temperature points were replicated in triplicates, except Day 0 for all temperatures for which n=6.

A sample was removed from the freezer, dissolved in 300 µL of 1:1 MtBE (methyl *tert* butyl ether)/MeOH (methanol) by first adding 150 µL of MtBE, sonicating for 15 seconds, and then adding 150 µL of MeOH. The solution was microcentrifuged (Centrifuge 5424, Eppendorf, Hamburg, Germany) at 21:130 x *g* 300 µL to pellet any insoluble material and the 200 µL of the supernatant was placed into a 300 µL vial HPLC for analysis. Carotenoids were analyzed with slight modifications (as previously described by Cooperstone 2015) on an Alliance 2695 HPLC with a 996 diode-array detector (Waters Corp., Milford, MA, USA). The column for carotenoid separation was a C30 column (4.6×250 mm, 3 µm, YMC Inc., Wilmington, NC, USA) at 35 °C. Solvent A = 60% MeOH, 35% MTBE, 3% H_2O, 2% aqueous ammonium acetate, 0.05% TEA; Solvent B=78% MTBE, 20% methanol, 2% aqueous ammonium acetate (2% w/v), 0.05% TEA. The flow went as follows: initial conditions of 100% A, with a linear gradient to 50% A for the first 10 minutes, to 0% A over the next 5 minutes, held at 0% A for 3 minutes, then back to initial conditions over the next 3.5 minutes. The flow rate was 1.30 mL/min. The total run time was 21.5 minutes for each sample.

DATA ANALYSIS

A chromatogram was obtained from each sample, which measures the absorbance of compounds as they elude from the column. The peaks for eight key compounds at 450 nm were integrated: lutein, zeaxanthin, beta-cryptoxanthin, alpha-carotene, beta-carotene, other-*cis*-lycopene, all-*trans* lycopene, 5-*cis* lycopene. In addition, the total *cis*-lycopene, total lycopene, and total carotenoids were tabulated.

The level of each compound was determined employing authentic standard curves to convert peak area into concentration (nmol/L). A sample chromatogram can be seen in Figure 1.

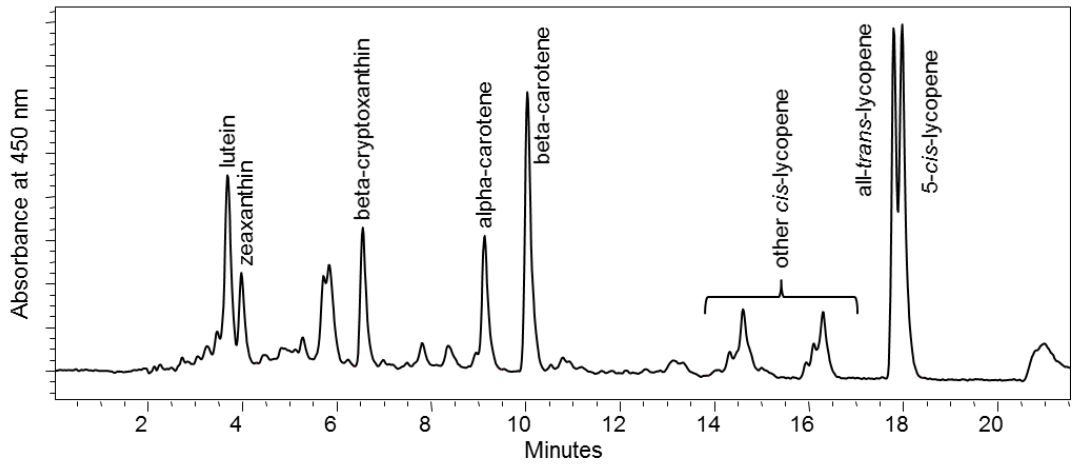

Figure 1. Chromatogram of carotenoids in blood plasma at 450 nm.

The carotenoid concentrations were statistically analyzed using a two-way ANOVA and Tukey's post-hoc test, modeling the effect of storage time, storage temperature, and the interaction of these two variables.

RESULTS AND DISCUSSION

We hypothesized that the levels of each carotenoid would decrease over time and the warmest storage temperature (4°C) would have the fastest rate of degradation. However, carotenoids extracted from plasma were more stable than expected. In several cases, there was not a significant difference in the amount from Day 0 to Day 120. While it is unlikely that projects would leave biological samples in storage for this long before analysis, it is beneficial to know that there is a long period of stability for most carotenoids analyzed. But while there was less degradation over time than expected, the most important variable in the stability of carotenoids was storage time. There was a significant effect of length of storage for each carotenoid and groups of carotenoids like "Total Lycopene". This supports the hypothesis that there is a relationship between storage time and the level of carotenoid in the plasma sample.

Table 1. Significant variables for carotenoids during cold storage

Significant effect of storage time	Lutein, Zeaxanthin, Beta-cryptoxanthin, Alpha-carotene, Beta-carotene, Other-*cis*-lycopene, All-*trans*-lycopene, 5-*cis*-lycopene, Total *cis*-lycopene, Total lycopene, Total carotenoids
Significant effect of storage temperature	All-*trans*-lycopene, Other-*cis*-lycopene
Significant effect of time: temperature interaction	Alpha-carotene, Beta-carotene, All-*trans*-lycopene, Other-*cis*-lycopene, Total *cis*-lycopene, Total lycopene, Total carotenoids

The storage temperature and the interaction between storage temperature and storage time was only significant in a few of the compounds, as seen in Table 1. Data analysis was performed at several time points during the project, and as more time had elapsed, more compounds had a significant effect of time by temperature interaction. This result supports the hypothesis that carotenoid stability is impacted by both the temperature and length of storage, and that both variables need to be taken into account when scheduling extraction and analysis. The carotenoids of interest will determine the appropriate storage temperature and time.

Figures of each carotenoid concentration over storage time and with varying temperature can be seen in the Appendix. The designations for significant differences are only shown for days, even for compounds that had significant effects of other variables. The levels between days and storage temperature did not often follow the expected pattern of the least amount in the 4°C fridge and less over time. Day 14 was consistently lower in the carotenoids analyzed but it is unclear why these levels were low. While there may be some isomerization between some carotenoids, it is otherwise not possible for the compounds to degrade and then reform while in storage. Current work is underway to investigate Day 14 effects.

CONCLUSION

This stability study showed that carotenoids are generally stable at -20°C or -80°C for up to 120 days. The storage time was the most significant variable, yet there was less degradation over the four-month period than expected. With this information, future studies can better plan extraction and HPLC analysis of samples. Because storage time is a significant factor for all carotenoids, samples should be analyzed quickly; however there is a longer window for analysis than expected because the levels were stable,

even 90 and 120 days out. The implications of this stability study will differ depending on the level of analysis for other studies; those looking at exact amounts of isomeric breakdown of lycopene (including the ratio of all-*trans*-lycopene to other *cis*-isomers) should store samples at lower temperature for a shorter time, as compared to an epidemiological study that may only need the total carotenoids level.

Future stability studies are needed to better verify the stability of carotenoids at other points in the extraction and analysis procedures. This may include the degradation rates while samples are in the auto-sampler in the HPLC machine, awaiting analysis. If a larger number of samples can be extracted and loaded at once, and perhaps even left to run overnight, there can be a higher through-put for high-volume studies.

APPENDIX

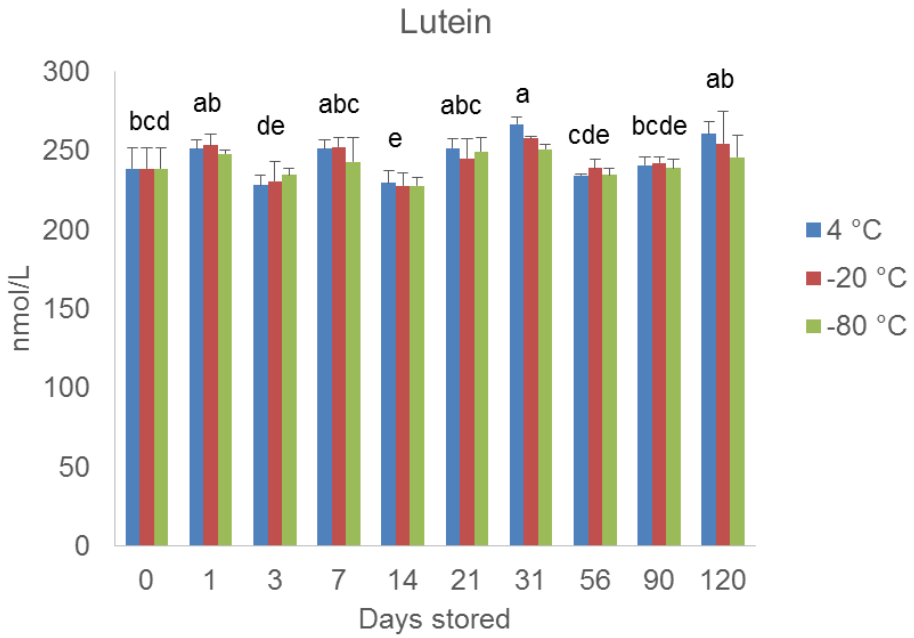

Figure 2. Post-extraction lutein stability during cold storage

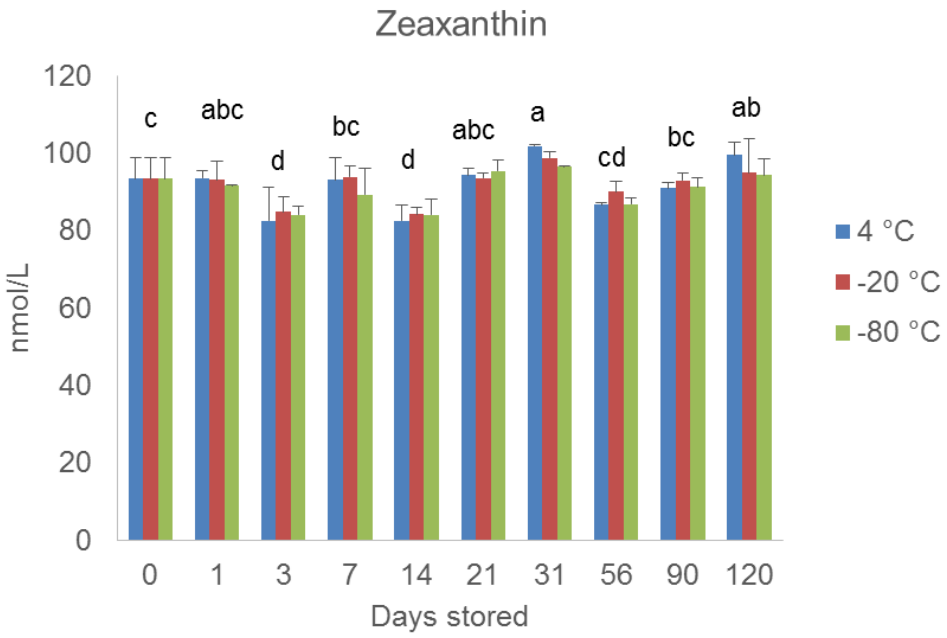

Figure 3. Post-extraction zeaxanthin stability during cold storage

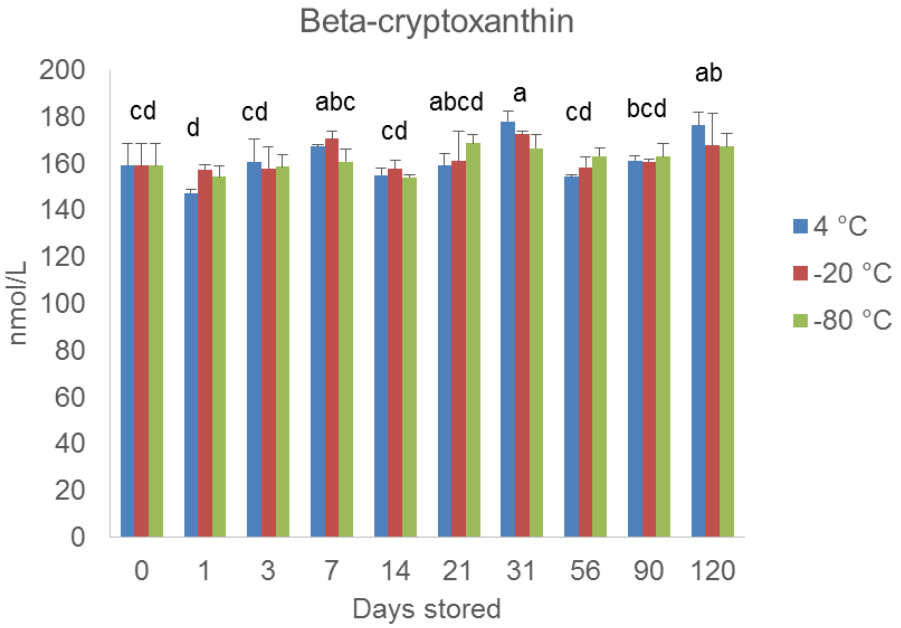

Figure 4. Post-extraction beta-cryptoxanthin stability during cold storage

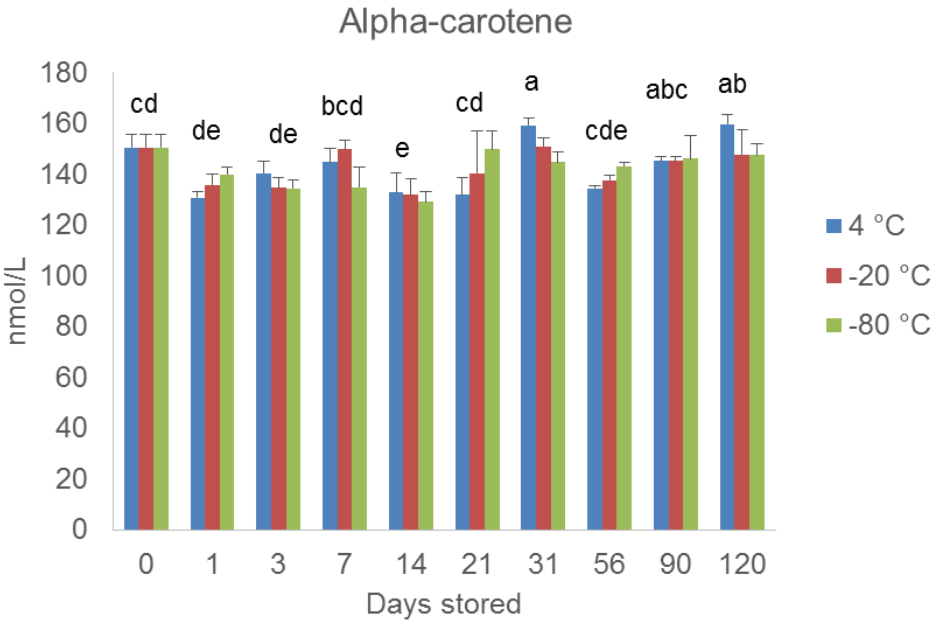

Figure 5. Post-extraction alpha-carotene stability during cold storage

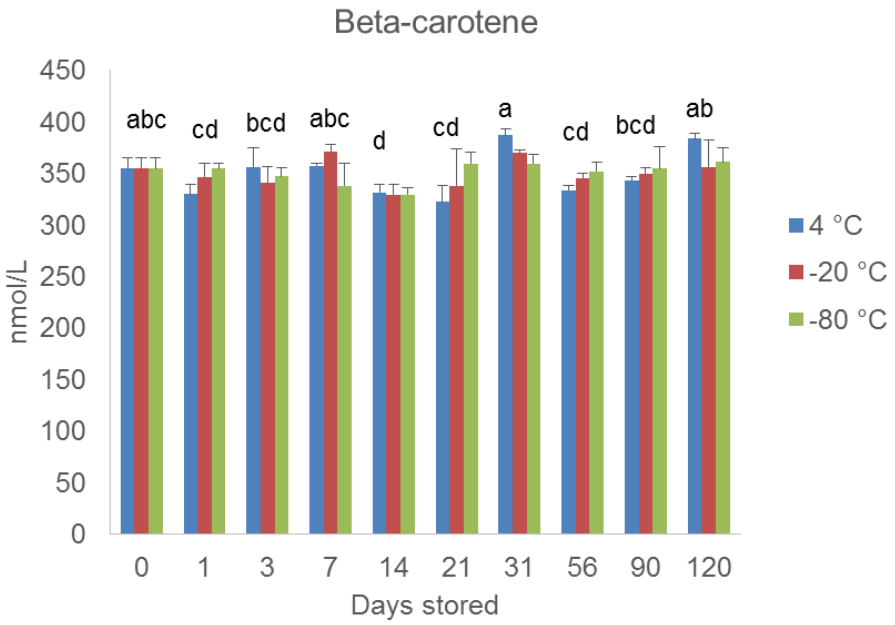

Figure 6. Post-extraction beta-carotene stability during cold storage

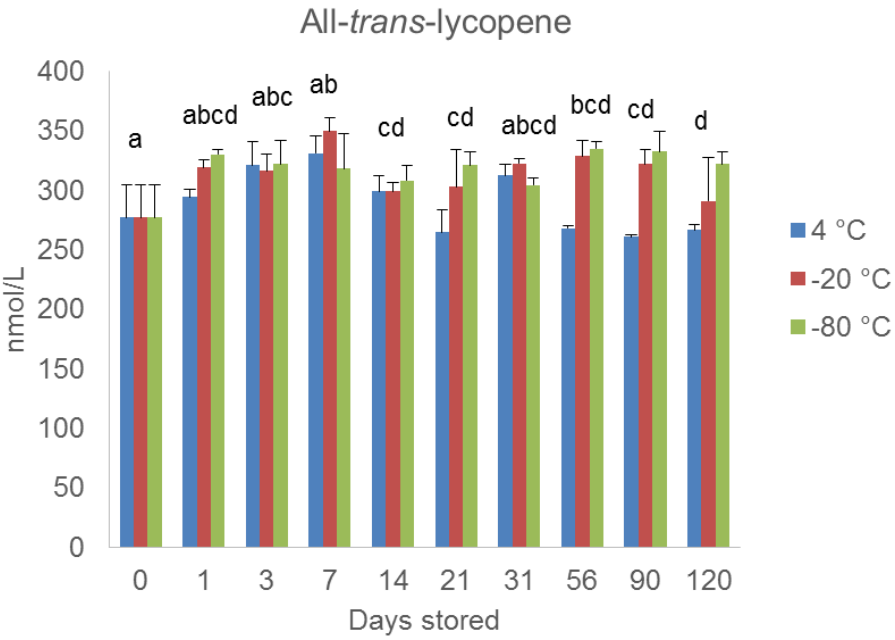

Figure 7. Post-extraction all-*trans*-lycopene stability during cold storage

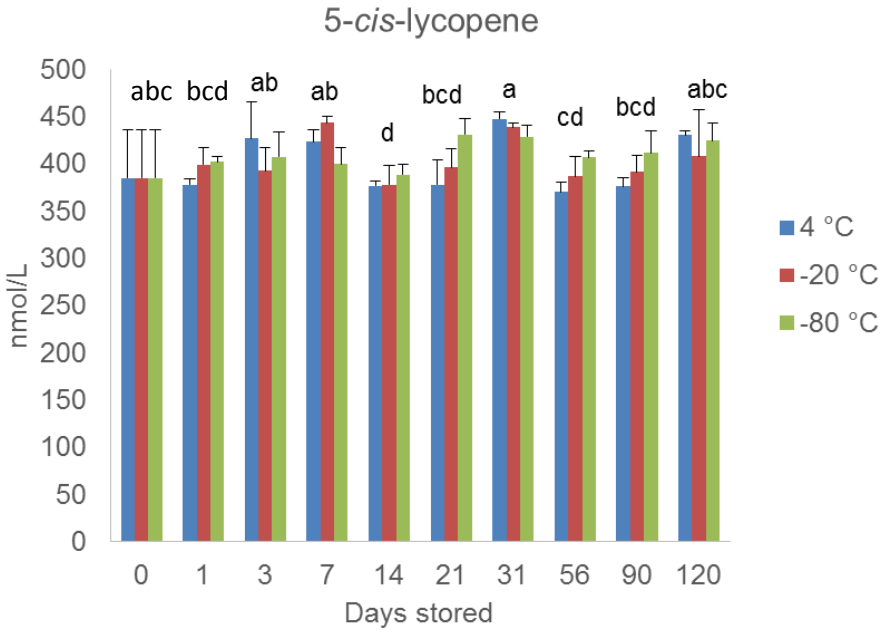

Figure 8. Post-extraction 5-*cis*-lycopene stability during cold storage

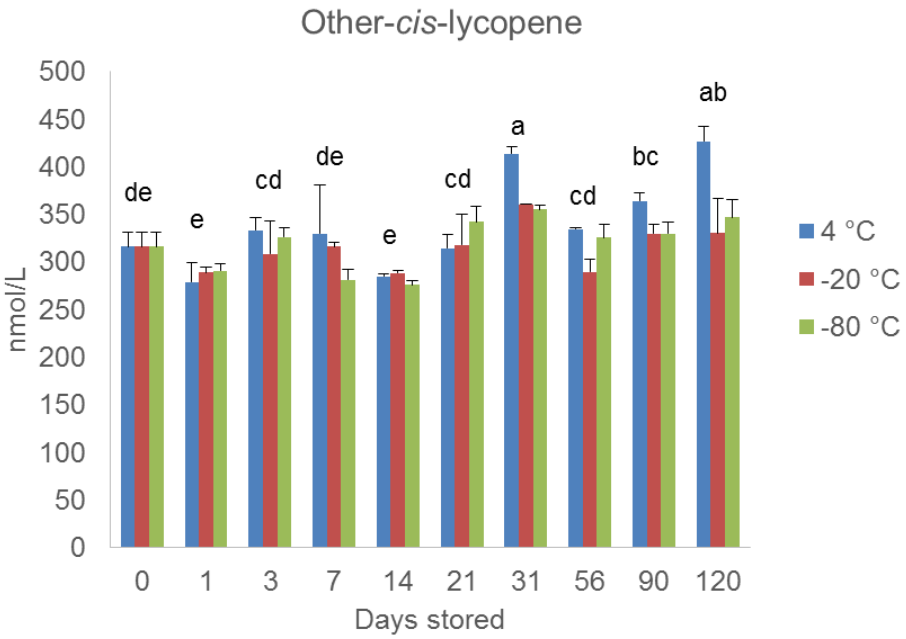

Figure 9. Post-extraction other-*cis*-lycopene stability during cold storage

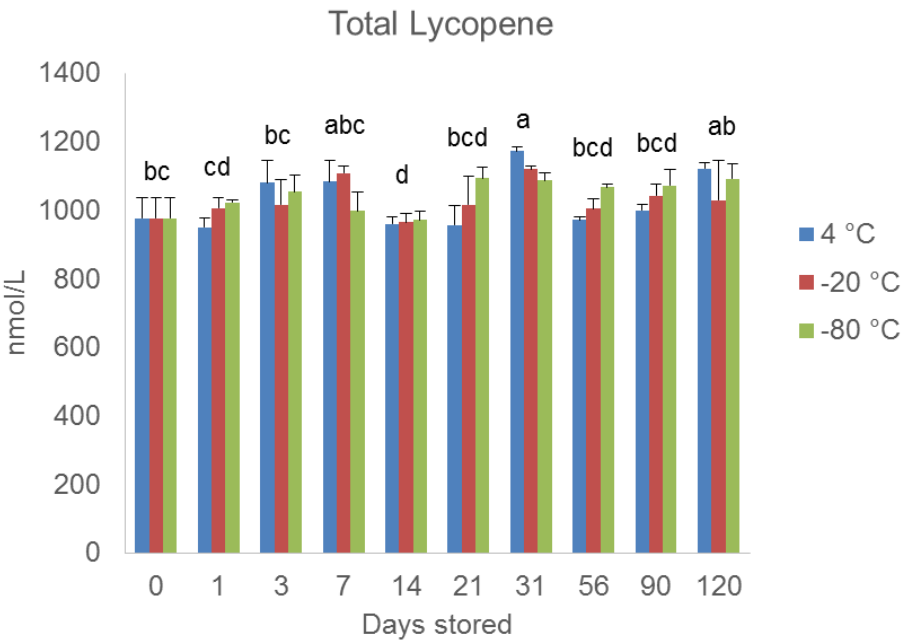

Figure 10. Post-extraction total lycopene stability during cold storage

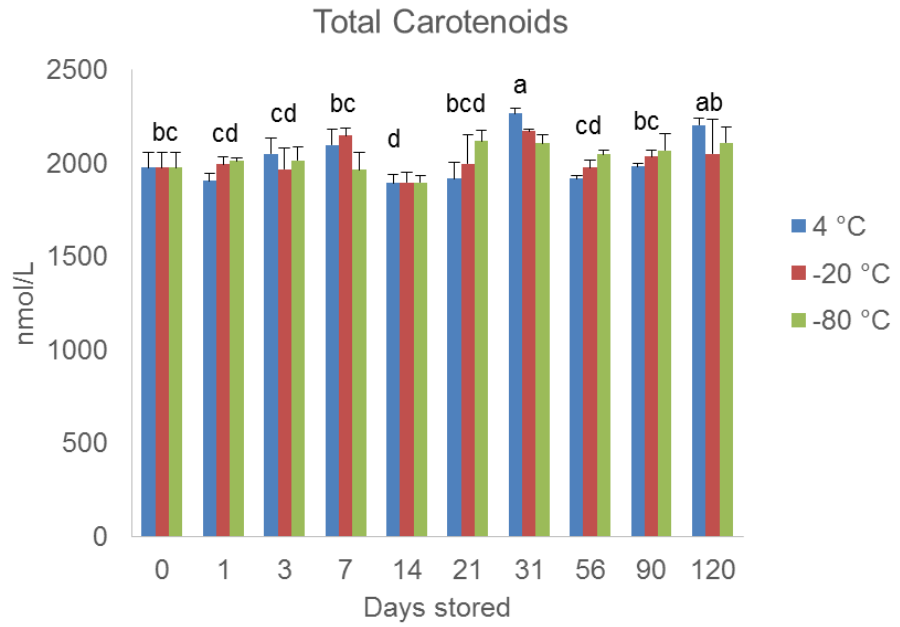

Figure 11. Post-extraction total carotenoid stability during cold storage

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